In Vitro and In Vivo Activities of WIN 54954, a New Broad-Spectrum Antipicornavirus Drug

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WIN 54954 (5-[5-[2,6-dichloro-4-(4,5-dihydro-2-oxazolyl)phenoxy]pentyl]-3-methylisoxazole) is a new member of the class of broad-spectrum antipicornavirus compounds known to bind in a hydrophobic pocket within virion capsid protein VP1. In plaque reduction assays, WIN 54954 reduced plaque formation of 50 of 52 rhinovirus serotypes (MICs ranged from 0.007 to 2.2 μg/ml). A concentration of 0.28 μg/ml was effective in inhibiting 80% of the 52 serotypes tested (EC80). WIN 54954 was also effective in inhibiting 15 commonly isolated enteroviruses, with an EC80 of 0.06 μg/ml. Furthermore, WIN 54954 was effective in reducing the yield of two selected enteroviruses in cell culture by 90% at concentrations approximately equal to their MICs. The therapeutic efficacy of intragastrically administered WIN 54954 was assessed in suckling mice infected with coxsackievirus A-9 or echovirus type 9 (Barty) 2.5 days prior to initiation of therapy. Single daily doses of 2 and 100 mg/kg protected 50% of the mice from developing paralysis (PD50) following infection with coxsackievirus A-9 and echovirus 9, respectively. At the PD50 doses for these two viruses, levels of WIN 54954 in serum were maintained above the in vitro MICs for a significant portion of the dosing interval. The dose-dependent reduction in viral titers observed in coxsackievirus A-9-infected mice correlated well with the therapeutic dose response. The potency and spectrum of WIN 54954 make it a potentially useful compound for the treatment of human enterovirus and rhinovirus infections.

Picornaviruses are a family of small, single-stranded RNA viruses, which includes a number of significant human pathogens. The rhinoviruses and enteroviruses are two groups within the Picornaviridae family which cause a range of human viral syndromes. Rhinoviruses are a major cause of mild upper respiratory infection in humans (8). The enteroviruses, which include the polioviruses, echorhoviruses, and coxsackieviruses, are associated with many syndromes, including poliomyelitis, myocarditis, aseptic meningitis, encephalitis, and upper respiratory tract infections (16). At present, no effective antiviral agents are commercially available for the treatment of the diseases caused by these viruses.

Previous reports have shown that the antipicornaviral agent disoxaril (WIN 51711) is systematically active in treating enterovirus infections in mice (9, 10, 12). This class of compounds has been shown to bind in a specific hydrophobic pocket within the capsid protein VP1 (4) and prevent viral attachment or uncoating, depending on the picornavirus serotype involved (13). Based on these findings, further chemical synthesis was undertaken in an effort to increase both the spectrum and potency of this class of antiviral compounds. WIN 54954 (Fig. 1) was synthesized (6) and, as described in this report, found to be a potent inhibitor of rhinovirus replication in vitro and of selected enteroviruses in vitro and in vivo.

MATERIALS AND METHODS

Media and solutions. The following media and solutions were used: minimal essential medium, medium 199 (M-199), and 2× M-199 (Flow Laboratories, Inc., McLean, Va.), newborn calf serum and fetal calf serum (FCS) from GIBCO Laboratories (Grand Island, N.Y.), 1% Seakem agarose in water (FMC Corp., Marine Colloids Div., Rockland, Maine), 3 mg of DEAE-dextran per ml (Sigma Chemical Co., St. Louis, Mo.; M, 500,000), 3 M MgCl2, 5% gluteraldehyde for fixing, and 0.25% crystal violet for staining. For plaque reduction assays, a set of 200× stock solutions of WIN 54954 were prepared in dimethyl sulfoxide (DMSO) and diluted in M-199 to achieve final concentrations of 0.001 to 6.2 μg/ml.

Cells and viruses. Coxackievirus A-9 was originally obtained from C. Wilfert, Duke University, N.C., and cultured in LLC-MK2 cells. Coxackievirus B-3 was originally obtained from C. Quanant, University of Texas, San Antonio, and cultured in Vero cells. Echovirus types 11 and 9 were obtained from the Wadsworth Center for Laboratories and Research, Albany, N.Y., and cultured in RD-2 cells. Echovirus type 9 (Barty) was originally obtained from Rockefeller University, New York, N.Y. A 10% suspension of tissue from suckling mice infected with echovirus type 9 (Barty) was made in M-199 medium. All other viruses were obtained from the American Type Culture Collection, Rockville, Md. Human rhinoviruses were cultured in HeLa (Ohio) cells. Echoviruses and coxsackieviruses were cultured in RD-2 cells. All cell cultures used in plaque reduction and virus yield reduction assays were grown in six-well 35-mm2 tissue culture plates (Costar, Cambridge, Mass.) in a 2% CO2 atmosphere.

Plaque reduction assay. Medium was aspirated from confluent monolayers of cells and cells infected with 1.0 ml of the appropriate virus (approximately 80 PFU per well) in M-199 medium. The cultures were incubated for 1 h at 37°C for enteroviruses or at 33°C for rhinoviruses. The virus inoculum was removed, and the cells were overlaid with M199 containing 5% newborn calf serum or 5% FCS, 30 mM MgCl2, 0.5% agarose, and WIN 54954 at various concentrations. The overlay for human-rhinovirus-infected cells also contained 15 μg of DEAE-dextran per ml. Enteroviruses

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were allowed to replicate (forming plaques) for 1 to 3 days at 37°C and rhinoviruses for 3 to 4 days at 33°C in a 2% CO₂ atmosphere. Cells were fixed with 5% glutaraldehyde and stained with 0.25% crystal violet. The concentrations of WIN 54954 which inhibited plaque formation by 50% were determined for each virus and recorded as the MIC. The MICs determined in this manner had a standard deviation of less than ±50%.

Viruses yield reduction assay. Confluent 1-day-old monolayers of RD-2 cells were infected with coxsackievirus A-9 or echovirus type 9 (Barty) at a multiplicity of 0.01 PFU per cell. WIN 54954 (suspended in 100× in DMSO) was diluted 100-fold in the virus inoculum at the indicated concentrations. After 1 h at 37°C and 2% CO₂, the inoculum was removed and the monolayers were washed once with medium containing the appropriate concentration of WIN 54954. Fresh medium (3 ml of M199 plus 5% FCS) containing WIN 54954 was added to each well, and the plates were incubated at 37°C for 16 h for coxsackievirus A-9 or 24 h for echovirus type 9 (100% destruction of cell sheet by the virus control) and then frozen at −70°C. The virus yield in each well was quantitated by plaque assay on monolayers of RD-2 cells. No effort was made to remove drug before progeny virus was plaque assayed. Other experiments (data not shown) have demonstrated reversible binding of drug to virus. Thus, serial dilution into drug-free medium during quantitation by plaque assay dissociates drug from virus. In the plaque assay, the number of plaques followed a dilution response, indicating that drug carryover is not a problem.

Evaluation of cytotoxicity by measurement of cell growth. RD-2 cells were seeded in six-well tissue culture plates (Costar) with M-199 plus 5% FCS and grown for 24 h at 37°C and 2% CO₂ to result in 5 × 10⁵ viable cells per well. WIN 54954 was dissolved in DMSO at 100×; the final concentration was diluted 1:100 into M-199 plus 5% FCS. A 3-ml sample of medium containing WIN 54954 at the desired concentration, ranging from 0.3 to 66 μg/ml, was added to each well at day 0. Control wells contained medium with 1% DMSO alone. On days 1 through 3, triplicate wells for each drug concentration and controls were examined microscopically for obvious toxic effects. The growth medium was then removed and the cell layer was rinsed with 0.5 ml of a 1:1 mixture of 4 mM disodium EDTA and 0.25% trypsin, followed by incubation with 2 ml of this mixture for 10 min at 37°C. After this time, the cells were detached and separated by gentle agitation. Cell suspensions were counted in duplicate by trypan blue extension and a hemacytometer. By day 3, the untreated control cell number (1% DMSO) increased by approximately 10-fold above the day 0 level.

Mice. Swiss female albino ICR mice (Blue Spruce Farms, Altamont, N.Y.) that had delivered babies within the last 24 h were placed in plastic mouse boxes and provided with nesting material. The dams and pups arrived within 10 to 15. Each pup weighed between 1.2 and 1.9 g.

Infection of mice. Each mouse was infected subcutaneously with 0.03 ml of diluted virus over the right shoulder with a 0.5-ml Glaspak syringe and a 27-gauge, 0.5-in. (ca. 1.25-cm) needle. All suckling mice were infected within 24 h of birth. Coxsackievirus A-9 was diluted in phosphate-buffered saline to give 10 50% lethal doses (LD₅₀; 1 LD₅₀ equals 1 PFU per mouse). Echovirus type 9 (Barty) was diluted in phosphate-buffered saline to give 10 LD₅₀ (1 LD₅₀ equals 22 to 84 PFU per mouse).

Preparation of WIN compound, medication route, and regimen. WIN 54954 was prepared as a suspension in sterile 1% gum tragacanth (Fisher Scientific). Suckling mice were medicated intragastrically with the use of a 1-in. 24-gauge Perfectum animal feeding needle (Popper and Sons Biomedical Instrument Division, New Hyde Park, N.Y.) on a 0.5-ml Glaspak syringe. The WIN 54954 suspension (0.03 ml) or placebo (1% gum tragacanth) was administered once a day (q.d.) beginning 2.5 days postinfection and continuing for 5 days. Five days of medication have been shown to be sufficient (9). Longer medication regimens result in similar efficacy (data not shown). WIN 54954 is efficacious prophylactically (data not shown). The regimen used represents a therapeutic regimen, which is a stringent test of antiviral efficacy.

Observation. Mice were checked twice a day (b.i.d.) for evidence of flaccid limb paralysis. Paralyzed animals were noted, and the percentage of nonparalyzed animals was calculated for each dose level for each day of the test. The 50% protective dose (PD₅₀) values were calculated by probit analysis (Proc. Probit Subroutine in SAS User’s Guide; Statistics, 1982 ed., SAS Inc., Cary, N.C.).

Quantification of virus titers in suckling mice. Titration of coxsackievirus A-9 was performed on homogenized tissue from mice killed on day 5 after infection, when peak titers were reached in control animals (data not shown). Mice were medicated b.i.d. for 2.5 days beginning 2.5 days postinfection. Five mouse pups from groups medicated with 1.2, 3.7, 11, and 33 mg of WIN 54954 per kg per day or placebo were killed, and the carcasses were washed with sterile 0.85% NaCl. Ten percent suspensions of individual carcasses were made in M-199 plus penicillin (100 U/ml) and streptomycin (100 mg/ml) with a Dualix tissue grinder (Kontes, Vineland, N.J.). Homogenates were frozen and thawed and then centrifuged to remove debris. The supernatants were placed in ampules and stored at −70°C for use in a standard tissue culture plaque assay with RD-2 cells (9).

Quantification of WIN 54954 levels in serum in suckling mice. Three-day-old suckling mice were medicated with a single intragastric dose of WIN 54954 in 1% gum tragacanth at a dose of 2, 14, or 100 mg/kg. At 1, 2, 4, 8, and 24 h after administration, groups of 20 mice per time interval were exsanguiated. The blood was pooled and allowed to clot, and the serum was obtained by centrifugation. Serum levels of WIN 54954 were measured by gas chromatography with a Hewlett-Packard 5710A gas chromatograph equipped with a nickel-63 electron capture and linked to a computerized data acquisition system (Hewlett-Packard model 3357 LAS) for collection and processing of data.

RESULTS

Inhibition of human rhinovirus and human enterovirus plaque formation. WIN 54954 was a potent inhibitor of plaque production of 50 of the 52 human rhinoviruses tested (Table 1). The MICs ranged from 0.007 to 2.2 μg/ml. Only rhinovirus 5 and rhinovirus 8 were not susceptible to the compound. A concentration of 0.28 μg/ml was effective in inhibiting 80% of the 52 serotypes tested (EC₅₀). Plaque
TABLE 1. Anti-human rhinovirus activity of WIN 54954

<table>
<thead>
<tr>
<th>Human rhinovirus serotype</th>
<th>MIC (µg/ml)*</th>
<th>Human rhinovirus serotype</th>
<th>MIC (µg/ml)</th>
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<tr>
<td>36</td>
<td>0.007</td>
<td>67</td>
<td>0.11</td>
</tr>
<tr>
<td>2</td>
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</tr>
<tr>
<td>22</td>
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</tr>
<tr>
<td>11</td>
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<td>0.17</td>
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<tr>
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<td>ND&lt;sup&gt;a&lt;/sup&gt; (&gt;6.2)</td>
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<td>39 (strain T-39)</td>
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<td>5</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt; (&gt;6.2)</td>
</tr>
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</table>

*Defined as the concentration required to reduce plaque number by 50%. Values are the mean of at least two separate assays and a standard deviation of less than ±50%.

ND, Not determined.

formation of 15 commonly isolated enteroviruses was inhibited by WIN 54954 (Table 2). The MICs ranged from 0.004 to 0.43 µg/ml. The EC<sub>50</sub> for the enterovirus serotypes was 0.06 µg/ml.

Effect of WIN 54954 on virus yield. The effect of WIN 54954 on multiple rounds of replication of coxsackievirus A-9 and echovirus type 9 (Barty) is shown in Fig. 2. A dose of 0.003 µg/ml resulted in a 90% reduction in coxsackievirus A-9 yield. A dose 10 times this concentration reduced the virus yield by 10<sup>6</sup>-fold and completely inhibited viral multiplication. WIN 54954 at 0.14 µg/ml reduced echovirus type 9 (Barty) yield by 90%. The maximum yield reduction achieved was 10<sup>3</sup>-fold (99.6%) at 0.5 µg/ml.

RD-2 cell growth in the presence of WIN 54954. The in vitro cytotoxicity of WIN 54954 was determined by assessing the effects of the drug on RD-2 cell growth over a 3-day period in two separate experiments (data not shown). In each case, the cell number in the untreated control (1% DMSO) increased approximately 10-fold above the day 0 level. At concentrations of WIN 54954 of 2.5 µg/ml or lower, there was no effect (±11% inhibition) on cell growth at any time point (day 1, 2, or 3 after addition of WIN 54954). At 7 µg/ml, cell growth was inhibited by 50%, and at a concentration of 22 µg/ml, cell growth was essentially arrested.

Oral efficacy against coxsackievirus A-9 infection in suckling mice. Dose-dependent therapeutic efficacy of WIN 54954 in mice infected with coxsackievirus A-9 is shown in Fig. 3. The combined results of three dose-response studies are presented. The results are shown as the mean percent nonparalyzed animals on each day. Q.d. intragastric doses of WIN 54954 beginning 2.5 days after infection prevented the development of paralysis in mice in a dose-dependent manner. On day 13 postinfection, only 10% of the placebo-medicated animals were symptom free, compared with 36, 66, 87, and 95% of the mice medicated with WIN 54954 at doses of 1.2, 3.7, 11, and 33 mg/kg per day, respectively. From the data in Fig. 3, the PD<sub>50</sub> for WIN 54954 was determined to be 2.0 mg/kg per day q.d.

Effect of WIN 54954 on coxsackievirus replication in suckling mice. WIN 54954 prevented the replication of coxsackievirus A-9 in suckling mice in a dose-dependent manner.
FIG. 3. Oral therapeutic efficacy of WIN 54954 in coxsackievirus A-9-infected suckling mice. Mice were medicated q.d. for 5 days beginning 2.5 days postinfection with WIN 54954 at 1.2 (90 mice), 3.7 (89 mice), 11 (90 mice), and 33 (60 mice) mg/kg per day or with placebo (88 mice).

FIG. 4. Virus titers in coxsackievirus A-9-infected suckling mice medicated orally with WIN 54954 b.i.d. for 2.5 days beginning 2.5 days postinfection. Error bars indicate standard deviation.

FIG. 5. Oral therapeutic efficacy of WIN 54954 in echovirus type 9 (Barty)-infected suckling mice. Mice were medicated q.d. for 5 days beginning 2.5 days after infection with WIN 54954 at 3.7 (85 mice), 11 (88 mice), 33 (88 mice), and 100 (74 mice) mg/kg per day or with placebo (87 mice).

1 × 10⁵, 3.9 × 10⁵, and 1.6 × 10² PFU per mouse at WIN 54954 doses of 0, 1.2, 3.7, 11, and 33 mg/kg per day, respectively. To determine whether drug carryover in mouse tissue was responsible for the reduction in virus titers, homogenized samples from WIN 54954-medicated mice (33 mg/kg per day) were spiked with a known quantity of virus. All of the inoculated virus was recovered from these samples.

In vitro and in vivo activities of WIN 54954 against echovirus type 9 (Barty). WIN 54954 inhibited plaque formation of echovirus type 9 (Barty) by 50% at a concentration of 0.31 μg/ml [the MIC for echovirus type 9 (Barty), a mouse-adapted virus, is different from the MIC for echovirus type 9 (ATCC) shown in Table 2]. The dose-dependent oral therapeutic efficacy of WIN 54954 in suckling mice infected with echovirus type 9 (Barty) is shown in Fig. 5. The combined results of three dose-response studies are presented. Reversal of paralysis was seen in all dose groups, including placebo. Survival data on the day that the dose group showed the fewest number of symptom-free mice were used to assess efficacy (worst-case analysis). Under these conditions, 7% of the placebo-treated mice were asymptomatic, compared with 10, 10, 33, and 55% of the mice medicated with WIN 54954 at doses of 3.7, 11, 33, and 100 mg/kg per day q.d., respectively. The PD₅₀ for WIN 54954 against echovirus type 9 (Barty) was determined to be 100 mg/kg per day q.d.

Serum WIN 54954 concentrations in suckling mice. Serum WIN 54954 levels in suckling mice medicated orally with 2, 14, or 100 mg/kg are shown in Fig. 6. Maximum concentra-
tions in serum were 0.12, 0.79, and 3.41 μg/ml for the 2-, 14-, and 100-mg/kg dose groups, respectively. The apparent half-life (t_{1/2}) values ranged from 2.4 h at 2 mg/kg to 4.9 h at 14 mg/kg and to 8.8 h at 100 mg/kg. The areas under the serum concentration-time curve (AUC) were 0.48, 8.08, and 36.09 μg · h/ml for the 2-, 14-, and 100-mg/kg doses, respectively.

**DISCUSSION**

The data presented here demonstrate the in vitro and in vivo efficacy of WIN 54954, a new broad-spectrum antipicornavirus drug. Compared with disoxaril, the previous compound in this chemical class, a greater than 20-fold improvement in spectrum and potency was observed against 52 human rhinovirus serotypes, as evidenced by the drop in EC_{50} from >6.2 μg/ml for disoxaril to 0.28 μg/ml for WIN 54954 (data not shown). WIN 54954 was also effective in inhibiting 15 commonly isolated enteroviruses, with an EC_{50} of 0.06 μg/ml. Against two selected enteroviruses, WIN 54954 was effective in reducing the yield of virus in RD-2 cells by 90% at concentrations equal to or below the MICs. Replication of coxsackievirus A-9, a susceptible virus with an MIC of 0.004 μg/ml, was completely inhibited at 10 times the MIC. This antiviral activity is specific and not due to cytotoxicity, since a dose of 2.5 μg of WIN 54954 per ml had no significant effect on RD-2 cell growth. The specificity was expected because this class of compounds bind in a specific site within virion capsid protein VP1 (4). The hydrophobic compounds may also associate with cell membranes, but this association does not result in observable toxicity at antiviral concentrations. Mode of action studies indicate that WIN 54954 inhibits replication of human rhinovirus 14 by blocking adsorption to cellular receptors (13). Disoxaril, the first-generation compound, was shown to have no effect on the adsorption of human rhinovirus type 2 and poliovirus type 2 to host cells but has been shown to inhibit rhinovirus type 2 and poliovirus type 2 uncoating events (7, 17). Additional studies are in progress to determine whether WIN 54954 can inhibit uncoating as well as adsorption of human rhinovirus 14.

The therapeutic efficacy of WIN 54954 demonstrated in suckling mice infected with coxsackievirus A-9 and echovirus type 9 (Barty) indicates that the compound is sufficiently bioavailable when administered orally to prevent the development of faccic limb paralysis due to severe polymyositis and striated muscle necrosis (5, 11). Q.d. intragastric doses of WIN 54954, initiated 2.5 days after coxsackievirus A-9 infection, prevented the onset of paralysis in ≥90% of the mice pups at doses as low as 11 mg/kg per day, and doses of only 2 mg/kg per day protected 50% of the animals. Virus titers in carcasses of coxsackievirus A-9-infected suckling mice medicated with WIN 54954 were reduced in a dose-dependent manner and correlated well with the survival curves, indicating that the protective effects of WIN 54954 were related to the ability of the drug to inhibit virus replication in mouse tissue. At 3.7 mg/kg per day, the dose at which ≥50% of the animals were protected (PD_{50}), a 99% reduction in virus titers was observed. At higher doses of WIN 54954, 10^{-1} to 10^{-2}-fold reductions in virus titers resulted in ≥90% protection. These findings suggest that there is a threshold titer for coxsackievirus A-9 beyond which symptoms become evident. WIN 54954 administered after the establishment of infection is able to maintain virus titers below this threshold.

WIN 54954, administered to echovirus type 9 (Barty)-infected suckling mice with a regimen identical to that used against coxsackievirus A-9, resulted in a significantly higher PD_{50} of 100 mg/kg per day. These results are consistent with the higher MIC of WIN 54954 for echovirus type 9 (0.31 μg/ml) than for coxsackievirus A-9 (0.004 μg/ml). Also, in vitro yield reduction assays, WIN 54954 completely repressed coxsackievirus A-9 viral multiplication at a concentration of 0.03 μg/ml. Maximum inhibition of 10^{-2}-fold was observed with echovirus type 9 (Barty) at 0.5 μg/ml.

Determination of WIN 54954 levels in serum at the PD_{50} doses of 2 and 100 mg/kg indicated that levels in serum were maintained above the in vitro MICs for a portion of, and possibly over the whole, dosing interval. At a dose of 100 mg/kg, levels in serum at 24 h postmedication were 0.4 μg/ml, while the MIC for echovirus type 9 (Barty) was 0.31 μg/ml. A dose of 2 mg/kg resulted in levels in serum falling below the minimum quantifiable level of 0.02 μg/ml by 8 h postmedication. It is possible, however, that levels in serum remained above the MIC of 0.004 μg/ml for coxsackievirus A-9 for most of the dosing interval. These results suggest that to demonstrate efficacy in these models, levels of WIN 54954 in serum must be above the in vitro MIC for at least a portion of the dosing interval.

WIN 54954 has been shown to be nonmutagenic in the Ames test, the human lymphocyte assay, and the Chinese hamster ovary HG phosphoribosyltransferase forward mutation assays. As expected, this virus-specific inhibitor has low acute toxicity, with oral 50% acute lethal doses of 4 g/kg in rats and mice. One-month chronic toxicity studies in monkeys and rats have shown that daily doses of 100 and 160 mg/kg, respectively, were generally well tolerated (R. Fabian, personal communication).

A number of compounds have in vitro activity against rhinovirus mediated through direct binding to the virus capsid. Dichloroflavan, Ro 09-0410/Rob 09-0415, RMI 15731, and 44,081 R.P. have all been shown to have in vitro
antirhinovirus activity but have lacked clinical efficacy (14). New compounds Ro 09-0881, Ro 09-0535, and Ro 09-0696 (Roche Laboratories) have been shown to be more active than the earlier chalcone Ro 09-0410 (2). Another new antirhinovirus compound, R-61837, has potent in vitro activity and was effective in a prophylactic, double-blind, placebo-controlled clinical trial in human volunteers (1, 3). The finding of these new more potent antirhinovirus compounds, including WIN 54954, may lead to future clinical candidates for the control of rhinovirus infection in humans.

The increased potency and broader spectrum of activity of WIN 54954 represent a significant advance over disoxaril, the first compound in this series. WIN 54954 has been shown to be absorbed when given orally and efficacious against systemic enteroviral diseases in mice and represents a candidate for clinical evaluation in the treatment of human picornaviral infections.

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LITERATURE CITED